Differential Inhibition of Tonoplast H^+-ATPase Activities by Fluorescamine and Its Derivatives

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ABSTRACT

Corn (*Zea mays* L.) root tonoplast vesicles were treated with the primary-amine specific reagent, fluorescamine (FL). Modification by FL caused a differential inhibition to the coupled activities of tonoplast H^+-ATPase. Within the range of 0 to 5 micromoles of FL per milligram of protein, the proton pumping rate was significantly reduced but ATP hydrolysis was only slightly affected. Yet, the membrane H^+ leakage during the pumping stage increased only slightly. FL treatment resulted in (a) a decrease in amine containing phospholipids and (b) an insertion of multiple H-bonding moieties into the membrane. To test which of these two possible effects were responsible for inhibition, FL derivatives of benzylamine, butylamine, and phenylalanine were synthesized. It was found that the acyclic derivatives with high H-bonding potential at concentrations of 10 micromolar inhibited proton pumping by 50% without a significant effect on ATP hydrolysis. Cyclic derivatives were largely ineffectual. Proton leakage during pumping was not affected by these acyclic modifiers. Membrane fluidity, as measured by the polarization of diphenyl hexatriene, decreased upon treatment with either FL or its derivatives. The results suggest that the proton pumping is indirectly linked to ATP hydrolysis in the tonoplast vesicles, and the link between these processes is apparently weakened by the presence of acyclic fluorescamine derivatives in the membrane.

The tonoplast membrane of plant roots contains at least two enzymes, an ATPase and a pyrophosphatase, which can convert the chemical free energy released from the hydrolysis of high-energy phosphoester bonds, into a transmembrane proton, electrochemical gradient (2, 6, 8, 33). According to the chemiosmotic concept, the resulting pH gradient and membrane potential may be used as the primary driving force to transport materials across the membrane (15, 19). Indeed, the coupling of the proton gradient to the movement of Ca^{2+} (21, 22) and sucrose (5) has been reported for tonoplast membranes. However, the molecular mechanism by which ATP hydrolysis induces vectorial proton transport remains unresolved.

The relative consistency of H^+/ATP ratio for the tonoplast H^+-ATPase obtained under certain conditions would suggest that the coupling between ATP hydrolysis and proton pumping is direct (1). The direct coupling mechanism implies that at least one molecular event must be common to the pathways leading to ATP hydrolysis and proton pumping (14). Coupling could also be accomplished through an indirect linkage, such as a membrane Bohr effect advocated by some researchers (10, 28). In our previous work with corn root tonoplast vesicles, we demonstrated that proton pumping exhibited a greater sensitivity to nitrate inhibition (29), temperature (27), mercury (27), and divalent cations (30), as compared to ATP hydrolysis. To further explore the nature of the coupling between these two processes, we used fluorescamine modification employed in previous studies of mitochondria and purple membrane systems (13, 28, 31).

Fluorescamine (Fig. 1, structure I) is an irreversible, primary-amine-specific fluorescent reagent (32, 34). The reaction is rapid and nearly quantitative at neutral or alkaline pH at room temperature (25). The excess reagent, if any, reacts relatively fast with water to generate nonfluorescent products. The reaction products (compounds II) are highly fluorescent and contain hydroxyl, carboxyl, and carbonyl groups which are excellent H-bonding functionalities. The acyclic fluorescent products slowly recyclize to form compounds III which have lower H-bonding capability (20, 28).

As described in previous studies, direct covalent modification of mitochondrial membrane (13) or *Halobacterium* membrane (31) with I resulted in a preferential inhibition of energy-driven (electron transfer, ATP hydrolysis, and light-dependent) proton pumping. A similar preferential inhibition of proton pumping was also observed in the presence of acyclic (compound II) but not cyclic derivatives (compound III) (28). Thus, the presence of H-bond forming groups in the membrane was essential for inhibition. These results suggested that the primary energy yielding process was only indirectly linked to proton pumping in those systems. Furthermore, the linkage appeared to be sensitive to the insertion of acyclic derivatives (compound II) in the membranes.

In the present study, we report the effects of fluorescamine and its derivatives on the coupled activities of the H^+-ATPase in tonoplast vesicles obtained from corn roots. The results suggest that the ATP hydrolysis step is linked to proton pumping through a molecular arrangement which is sensitive to the presence of acyclic fluorescamine derivatives (compound II) in the membrane. A possible model to explain these observations is proposed.

MATERIALS AND METHODS

Plant Materials

Corn seeds (*Zea mays* L. cv FRB 73, Illinois Foundation Seeds[1]) were germinated and harvested as previously described

[1] Reference to brand and firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

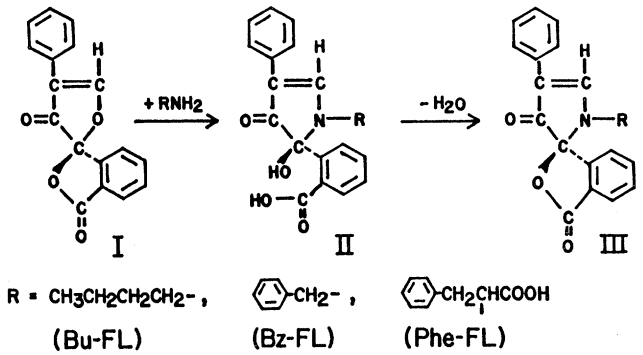

Figure 1. Reaction between fluorescamine and primary amines.

(29). Approximately 60 to 80 g fresh weight of excised roots were homogenized with mortar and pestle at 4°C in a homogenizing butter containing 0.3 M sucrose, 5 mM EDTA, 5 mM 2-mercaptoethanol, 5 mM DTE, and 0.1 M Hepes (pH 7.7) (adjusted at 4°C). The crude homogenate was filtered through four layers of cheesecloth. Differential centrifugation at 6,000*g* for 20 min and 80,000*g* for 35 min was performed to obtain a microsomal pellet. Microsomes were resuspended in 20 to 26 mL of homogenizing medium and centrifuged again at 80,000*g* for 35 min. Washed microsomes were suspended in grinding medium and overlaid on a 15 to 45% (w/w) linear sucrose gradient buffered with 5 mM Hepes-Mes (pH 7.7) plus 1 mM DTE and centrifuged at 4°C for 18 h at 84,000*g* (average) in a Beckman SW-28 rotor as described previously (29). The tonoplast fractions between 19 and 23% sucrose were pooled and used for the analyses. Tonoplast vesicles so isolated from roots of cultivar FRB73 are of very high purity because all of other membranes sediment as one peak with an average density of 38% sucrose (29).

Modification of Tonoplast Vesicles

Two hundred μL aliquots of purified tonoplast vesicles (20–40 μg of protein) were warmed to room temperature over 3 min. While vortexing, up to 2 μL of acetone, typically containing 10 mM fluorescamine, were rapidly added and then mixed for 5 s. Immediately after the vortexing, the vesicles were diluted to 2.0 mL with the proton pumping assay medium. After standing at room temperature (22°C) for 6 min, the proton pumping and ATP hydrolysis were assayed as described below. Samples prepared by the addition of acetone only were used as controls. The effects of fluorescamine derivatives were determined in the same manner except the concentration of stock solutions in acetone was 33 mM.

Synthesis of Compounds II and III

The fluorescamine derivatives (II) of benzylamine, butylamine, and phenylalanine were synthesized and purified as described by de Bernardo *et al.* (9) and Purcell *et al.* (18). The purity of the derivatives was established by HPTLC[2] and IR spectroscopy as described previously (20). The derivatives were stored in the dark at −20°C as stock solutions in acetone and used promptly. The cyclization of derivatives II to yield the corresponding lactones (III) was accomplished as mentioned by Ramirez *et al.* (20).

Measurement of ATP Hydrolysis

ATP hydrolysis was assayed by the direct measurement of inorganic phosphate released at 22°C as previously described (29). The release of inorganic phosphate was determined in 2.2 mL of the basal solution containing 50 mM KCl, 7.5 μM acridine orange, 17.5 mM Mes (pH 6.45), 2.5 mM $MgSO_4$, and tonoplast vesicles. The reaction was started by the addition of 20 μL of 0.2 M ATP and terminated after 6 min. During this reaction time, the rate of ATP hydrolysis was constant (29). The amount of inorganic phosphate released was determined by the malachite green assay. At least 95% of the ATP hydrolysis is catalyzed by the tonoplast ATPase based on the sensitivity to nitrate, vanadate, azide, and molybdate (29). Parallel assays were conducted either in the presence of 50 mM KNO_3 or the absence of Mg to assess the effects of different treatments on the minimal ATP hydrolysis of non-tonoplast origin.

Proton Pumping

The proton movement associated with tonoplast vesicles, as measured by changes in the absorbance of acridine orange at 492.5 nm (29), was analyzed by the following equation:

$$d\delta/dt = R_0 - k_1\delta \quad (1)$$

in which $d\sigma/dt$ represents the net proton transport rate at time t after the addition of ATP, R_0, the initial proton pumping rate, k_1, a rate constant measuring energized membrane leakage and other possible inhibition processes such as back pressure (35) and pump slippage (17), and δ, the extent of proton transport at time t. At steady state, the net transport rate becomes zero. Thus,

$$R_0 = k_1\delta_s \quad (2)$$

in which δ_s is the extent of proton transport at the steady state. Equation 2 may be integrated to yield

$$\delta = \delta_s(1 - \exp[-k_1 t]) \quad (3)$$

or

$$\ln(1 - \delta/\delta_s) = -k_1 t \quad (4)$$

Since the proton transport is expressed by the absorbance change, Equation 3 can be rewritten as

$$(A_t - A_i) = (A_s - A_i)(1 - \exp[-k_1 t])$$

or

$$A_t = A_s - \Delta A^* \exp(-k_1 t) \quad (5)$$

in which A_i, A_t, and A_s represent the absorbance of the indicator at time 0, t, and steady state, respectively. If the change in fluorescence of a dye is used to measure proton

[2] Abbreviations: HPTLC, high performance thin layer chromatography; Bu, butylamine; Bz, benzylamine; DPH, 1,6-dipehnyl-1,3,5-hexatriene; FL, fluorescamine, or 4-phenylspiro[furan-2(3*H*),1′-phthalan]-3,3′-dione; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; P, polarization.

movement, then Equation 5 assumes the form of

$$F_t = F_s - \Delta F * \exp(-k_1 t) \quad (6)$$

As described in our previous report (30), an identical mathematical expression as Equation 6, was obtained by direct curve fitting (11). We have demonstrated previously (27, 29) that under experimental conditions, ATP hydrolysis remained constant during the buildup of the proton gradient in corn root tonoplast vesicles. Thus, the initial proton pumping rate, R_0, may be related to ATP hydrolysis rate by:

$$R_0 = mR_{ATP} \quad (7)$$

The quantity 'm' would then represent the stoichiometric ratio of H^+/ATP or a measure of the coupling between the two coupled processes. After obtaining the proton pumping information (1–2 min after reaching steady state), the same samples were used to determine the rate of ATP hydrolysis. All of the proton pumping at pH 6.45 is catalyzed by the tonoplast H^+-ATPase based on sensitivity to nitrate and vanadate (29). In addition, the vesicles contain very little pyrophosphate driven H^+ transport which is detected only at pH between 7.8 and 8.5 (data not shown).

The proton gradient at steady state (δ_s) could be discharged by a rapid depletion of substrate (Mg-ATP) by the addition of hexokinase and glucose. This discharge, in essence, represents the membrane proton leakage in the deenergized state. We demonstrated previously (29) that this process can be described by a simple first-order decay equation:

$$\ln(\delta/\delta_s) = -k_2 t \quad (8)$$

in which δ and k_2 represent the residual proton gradient and the leakage constant of deenergized membrane, respectively.

Membrane Lipid Extraction

Membrane lipids in 1.0 mL aliquots of tonoplast vesicles were extracted with organic solvent as described by Moreau and Isett (16). Polar and nonpolar lipids were separated simultaneously by HPTLC by a slight modification of the procedure of Kupke and Zeugner (12). Briefly, the organic solvent was removed at 60 to 70°C under a stream of N_2. The dried lipid film was redissolved in 30 to 100 μL of chloroform. Up to ten 1.0 μL aliquots were applied to a high performance silica gel LHP-K plate (10 × 10 cm) from Whatman. Plates were developed in 65:30:2.5 (by volume) chloroform:methanol:water until solvent front had migrated 3.5 cm from the origin. The plate was then air-dried and redeveloped in 80:20:1.5 (by volume) hexane:ether:acetic acid until the solvent front was within 0.5 cm of the top of the plate. After the plate was air-dried, lipids were then visualized and quantified by either fluorescent densitometry or charring followed by densitometry. The charring was accomplished by dipping the plate in a solution of 10% (w/v) $CuSO_4$ and 10% (w/v) H_3PO_4 and then heating at 120°C for 20 min. Both reflectance and fluorescent densitometry were determined using a CAMAG TLC Scanner II. The R_F values for PC, PE, and PS were 0.24, 0.47, and 0.54, respectively. The FL derivatives of both PS and PE exhibited a similar R_F value of 0.95.

Fluorescence Polarization Measurement

The effect of either direct fluorescamine modification or the presence of derivatives II on the structure of the tonoplast membrane was qualitatively estimated from the change in fluorescence properties of DPH incorporated into the membrane. The tonoplast vesicles, with or without modification, suspended in the proton transport medium without acridine orange were treated with 1.90 μM DPH for 20 min at 22°C. The suspension was then illuminated with vertically polarized light (370 nm) and the fluorescence intensities at the parallel (I_1) and the perpendicular (I_2) directions were measured at 424 nm with a Perkin-Elmer LS-5B Spectrofluorometer. P was calculated by the ratios of $(I_1 - I_2)/(I_1 + I_2)$.

Protein Concentration Measurement

The protein content of the tonoplast membrane vesicles was determined by a modified Lowry method using BSA as a standard (3). All the chemicals were obtained from commercial sources and were of analytical grade.

RESULTS

Establishing Experimental Conditions

Although FL has been applied to a variety of membrane systems in the past (13, 31), the exact conditions for optimizing the efficiency of labeling tonoplast vesicles needed to be established. The rate of FL hydrolysis in vesicle-free medium was studied first. When FL was injected into the proton assaying medium containing a simple amine, *e.g.* phenylalanine, a rapid increase in fluorescence which represented the formation of II was noted (data not shown). The reaction was essentially completed within 10 s. When FL was added to the medium, no fluorescence increase could be detected if the time between FL addition and addition of phenylalanine was longer than 5 s. Thus, FL was rapidly hydrolyzed to nonreactive, nonfluorescent products at pH 6.45, which was used in the present study. In the presence of tonoplast vesicles, the rate of labeling was slower and reaching completion 3 min after addition of FL. Apparently, the life time of the hydrophobic FL was increased in the membrane. Therefore, tonoplast vesicles were allowed to react with FL for 6 min before testing the functional effects of the modification.

The relative labeling efficiency was estimated by titrating a fixed amount of benzylamine with FL at different pH values. The labeling was followed by monitoring the increase in the absorbance at 390 nm. At pH 8.0, the labeling efficiency was determined to be 91%, in agreement with reported values (34). In the pH 6.45 medium used in the present work, this efficiency decreased to about 27%, probably due to increased hydrolysis of FL at the lower pH.

Effects of Fluorescamine Modification

FL reacted with the primary amine groups of tonoplast vesicles to yield fluorescent products. The extent of labeling

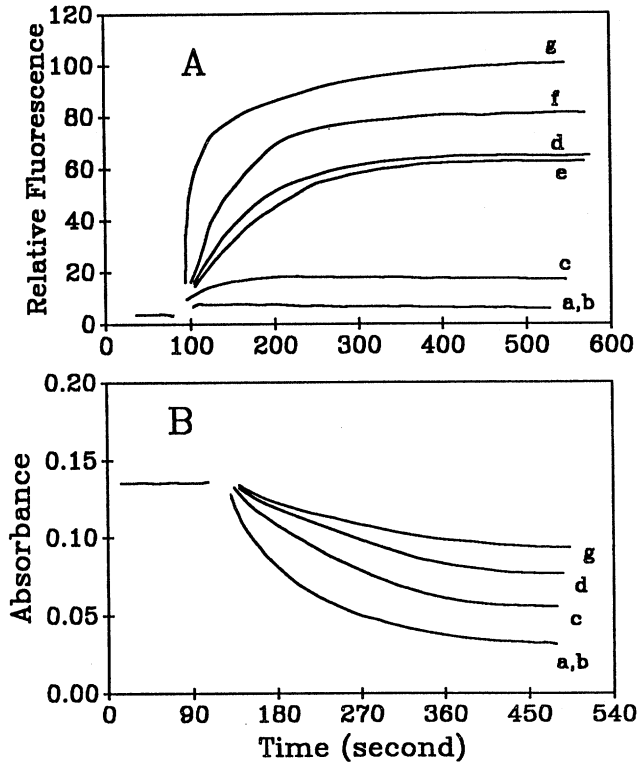

Figure 2. Fluorescamine labeling and its effects on proton pumping of tonoplast vesicles. Tonoplast vesicles were mixed for 5 s with fluorescamine stock solutions and then diluted with 1.8 mL of proton pumping assay medium without AO and the fluorescence was measured as a function of time (A). In a parallel experiment (B), treated vesicles were diluted in AO-containing medium, and incubated at 22°C for 6 min before the addition of ATP to induce proton pumping. For fluorescence measurements, the excitation and emission wavelength beams were fixed at 390 and 478 nm, respectively. The proton pumping was followed by the absorbance change of AO at 492.5 nm. Traces a, c, d, f, and g represent tonoplast vesicles (0.05 mg protein) treated with 0, 0.67, 3.33, 4.67, and 6.67 μmol fluorescamine/mg protein, respectively. In trace b, the identical amount of fluorescamine as used in d was first added to the medium and incubated for 6 min at 22°C before the addition of vesicles to determine the possible effects of the hydrolyzed products of fluorescamine. In trace e, vesicles were first diluted with the medium and then labeled with 3.33 μmol fluorescamine/mg protein.

could be qualitatively followed by fluorescence intensity increase at 478 nm (Fig. 2A). The extent of labeling was dependent on the concentration of FL added to the tonoplast vesicles. The proton pumping activity decreased as labeling increased (Fig. 2B). To assure that the observed inhibition was indeed due to modification, FL was allowed to be hydrolyzed before the addition of tonoplast vesicles. As shown by the traces A-b and B-b of Figure 2, neither an increase in fluorescence nor inhibition of proton pumping was detected when FL was incubated in the medium prior to the addition of tonoplast vesicles. These results indicate that the hydrolysis products of FL did not interfere with the functions of the H^+-ATPase.

The effect of modification to coupled Mg-ATP hydrolysis was compared to that of proton pumping. As depicted in Figure 3, labeling inhibited hydrolysis significantly less than proton pumping. The observed preferential inhibition of proton pumping was not the result of an interaction between the fluorescent label and the dye, acridine orange, used for monitoring proton movement. This was confirmed by the observation that neither the spectral properties, extinction coefficient and absorption maximum, nor the response of acridine orange to pH changes was affected by FL modification (data not shown). Similarly, the sensitivity and accuracy of the malachite green assay method for phosphate determination were not affected by the presence of FL modification (data not shown).

Origin of the Labels

Being a primary amine specific reagent, FL potentially could react with the N-terminus and lysine (ϵ-NH_2) residues of proteins and amine-containing phospholipids, such as PE and PS. Fluorescent derivatives were found in both organic and aqueous phases obtained from lipid extraction of treated tonoplast vesicles. As shown in Figure 3, the HPTLC analysis revealed that as the amount of FL was increased, the PE level decreased, and PE-FL increased almost parallel to the inhibition of proton pumping. However, using the same HPTLC analysis, no noticeable change of PS level was observed. Thus, it appears that the arrangement of PS in the tonoplast membrane significantly reduced its relative reactivity toward FL as compared to PE. When FL treated tonoplast vesicles were subjected to SDS-PAGE analysis, no apparent fluorescent polypeptide bands were detected. There was substantial fluorescence that comigrated with the tracking dye. However, this result does not rule out the possibility of a low level of labeling of membrane protein.

The above analyses on the origin of FL modification sug-

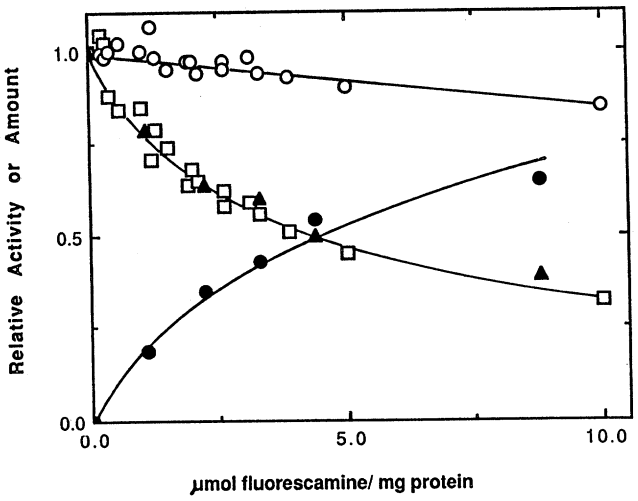

Figure 3. Differential effects of fluorescamine labeling on the coupled activities of the tonoplast H^+-ATPase. Corn root tonoplast vesicles were treated with different amounts of fluorescamine. The initial rates of ATP hydrolysis (○) and proton pumping (□) were determined as described. The data are plotted relative to controls (no fluorescamine treatment) which are 1.80 ΔA/min/mg protein for R_0 and 370 nmol Pi/min/mg protein for R_{ATP}. The contents of PE (▲) and PE-FL (●) were determined as described in text.

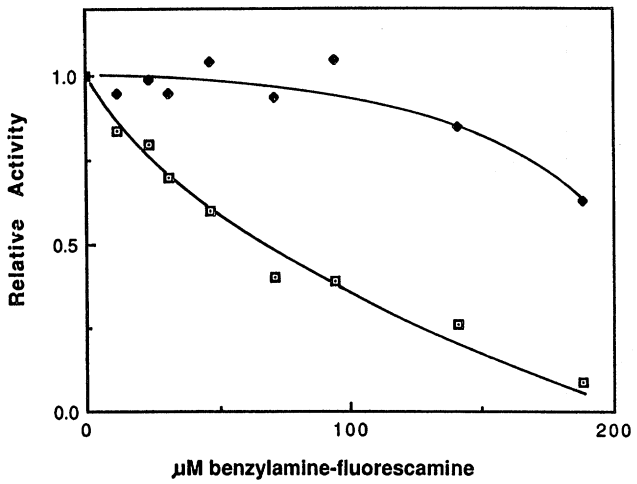

Figure 4. Effects of phenylalanine-fluorescamine derivative on coupled activities of the tonoplast H^+-ATPase. The derivative (compound II) was synthesized in aqueous acetone (80%) as described previously (20). The tonoplast vesicles were incubated with different concentrations of freshly prepared derivative for 6 min at 22°C before assaying of coupled activities. The data are plotted relative to controls which averaged 1.80 A/min/mg protein and 365 nmol Pi/min/mg protein for R_0 (□) and R_{ATP} (♦).

gested that primary amine groups both on proteins and membrane lipids could be important in regulating the coupled functions of the H^+-ATPase. However, it was equally plausible that the insertion of derivatized FL structure (II) into the membrane resulted in the inhibition observed in Figures 2 and 3. In this regard, the primary amine groups of PE could be considered as anchoring sites for the derivatized structure. Presumably, the labeled PE could diffuse laterally in the membrane phase such that the FL-accessible primary amines are not necessarily near the protein(s) involved in the coupling between ATP-hydrolysis and proton pumping. To test these possibilities, the effects of adding FL derivatives on the H^+-ATPase were performed.

Effects of Presynthesized Acyclic Derivatives

The activities of the tonoplast H^+-ATPase were analyzed in the presence of various concentrations of acyclic derivatives of FL with butylamine, benzylamine, and phenylalanine. In the presence of acyclic Bz-FL and Phe-FL, preferential inhibition of proton pumping was observed again (Figs. 4 and 5). The addition of 50 μM of the acyclic derivatives decreased the initial proton pumping rate (R_0) by 50% without any detectable influence on ATP hydrolysis rate. Similar results were also obtained with acyclic Bu-FL (data not shown). Thus, the destruction of membrane primary amine groups by FL could not be the main cause of observed preferential inhibition. While this result might suggest that these primary amine groups might not be important in the coupling mechanism, it does not rule out the possibility that certain FL inaccessible $-NH_2$ groups are involved in either ATP hydrolysis or proton pumping, or both.

The effects of the derivatives were compared to those caused by direct FL modification to evaluate the possible common interactions with the membrane. As summarized in Table I, detailed kinetic analysis revealed that the covalent and noncovalent treatments shared many common features. The presence of these modifiers increased membrane leakage toward protons (k_2) under deenergized conditions. Proton leakage during the buildup of proton gradient (k_1) was affected far less than k_2. Values of k_1 tended to increase slightly with modification (less than 40%). These results implied that the initiation of proton pumping by ATP hydrolysis induced certain rearrangement of membrane components to minimize scalar proton leakage during the buildup of the vectorial proton gradients. Since ATP hydrolysis was not affected by the treatment and proton leakage should be null at time zero, the decrease of R_0 could only be achieved by a reduction in m, the coupling between proton pumping and ATP hydrolysis. Thus, direct FL modification as well as the addition of FL derivatives inhibited the response of proton pumping mechanism to events occurring at the catalytic site of ATP hydrolysis.

Structural Requirement of Effective Derivatives

The results shown in Figures 4 and 5 demonstrate that the presence of acyclic structure of II in the membrane caused preferential inhibition of proton pumping. However, it remained to be determined which particular moiety of the FL derivatives was critical for this effect. A close examination of the acyclic structures of derivatives II points to the presence of hydroxyl, carboxyl, and carbonyl groups. These functional groups were known to be involved in the formation of hydrogen bonds in biological systems. It is possible that the presence of these functional groups at or near the proton conducting pathway of the pump may affect the coupled activities. This expectation agrees with the suggestion that intra-molecular

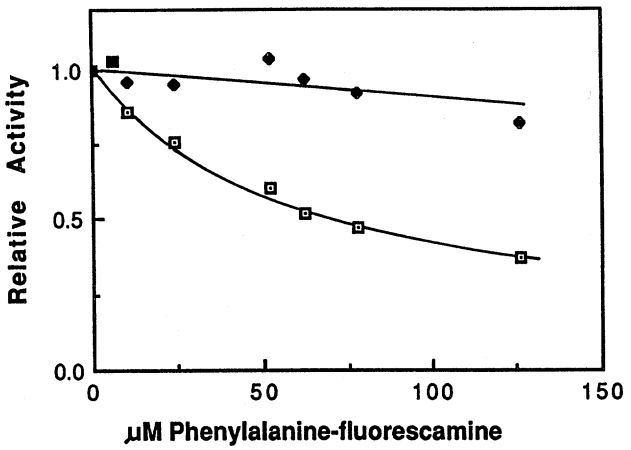

Figure 5. Effects of benzylamine-fluorescamine derivative on coupled activities of the tonoplast H^+-ATPase. Fluorescamine derivative of benzylamine was synthesized in acetonitrile as described in "Materials and Methods." The treatment of the tonoplast vesicles with freshly prepared derivative was similar to that described in Figure 3. Data are plotted relative to controls which averaged 1.75 ΔA/min/mg protein and 275 nmol Pi/min/mg protein for R_0 (□) and R_{ATP} (♦).

Table I. *Effects of Fluorescamine and Its Primary Amine Derivatives (II) on the Kinetic Parameters of Coupled Activities of the H^+-ATPase in Corn Root Tonoplast Vesicles*

Vesicles were either reacted with FL or incubated with cyclic FL derivatives with phenylalanine (Phe-FL), benzylamine (Bz-FL), or butylamine (Bu-FL) and then assayed for ATP hydrolysis, proton transport, and DPH fluorescence polarization (P) as described in "Materials and Methods." The values listed are relative to control (no fluorescamine or derivatives). The control values of R_O, R_{ATP}, k_1, k_2, and P are 1.75 ΔA/min/mg protein, 360 nmol Pi/min/mg protein, 0.93 min^{-1}, 0.65 min^{-1}, and 0.11, respectively. The data represent an average of three experiments with an error range as ±5%. Values for k_2 were determined by depleting ATP by the addition of hexokinase in the presence of glucose.

Reagent	ATPase Activities				DPH Fluorescence[a]
	R_O	R_{ATP}	k_1	k_2	P^2
None	1.00	1.00	1.00	1.00	1.00
1 μmol FL/mg protein	0.69	1.02	0.96	1.25	
2 μmol FL/mg protein	0.57	0.96	1.05	1.71	
4 μmol FL/mg protein	0.47	0.88	1.10	2.03	1.36
35 μM Phe-FL	0.63	0.97	0.97	2.22	
70 μM Phe-FL	0.40	0.87	1.31	3.82	1.64
35 μM Bz-FL	0.58	1.00	1.20	2.77	
70 μM Bz-FL	0.44	0.94	0.95	3.22	1.32
70 μM Bu-FL	0.80	1.06	1.00	1.60	1.10

[a] The concentration of DPH used was 1.9 μM.

H-bonding between helices of bacteriorhodopsin may be critical for allowing light-induced proton movement (26).

In previous studies of proton pumping in mitochondria (28), we demonstrated that the addition of cyclic derivatives (III) had no detectable effects on coupled activities. To determine if these hydroxyl and carboxyl groups of the acyclic derivatives (II) were essential for inhibition, the effects of cyclic derivatives III were compared to those induced by acyclic derivatives II. The cyclization of derivative II to III shifted the absorption maximum to a longer wavelength and profoundly reduced the fluorescence emission (Fig. 6). Although acyclic Bz-FL shared similar spectroscopic properties to Phe-FL (II), the cyclization of the former produced different changes. The ring formation of Bz-FL (II) shifted the absorption as well as the emission maximum to shorter wavelength (blue shift by about 10 nm). In addition, the decrease in emission intensity of cyclic Bz-FL was only about 50%. However, the addition of cyclic derivatives to tonoplast vesicles up to 100 μM had no detectable effects on the coupled activities of the H^+-ATPase (data not shown). Because of enhanced hydrophobicity, the partitioning of cyclic III into the membrane phase should be higher than that of acyclic II. The ineffectiveness of cyclic III can not be due to a lacking of membrane solubility. The preferential inhibition of proton pumping by II, but not III, suggests that intra- or intermolecular H-bonding of the tonoplast membrane components might be involved in the pathway of transmembrane proton pumping.

Effects of the Treatment on Membrane Fluidity

The relative fluidity of membranes could be qualitatively related to the polarizability (P) of incorporated fluorescent compound, DPH (23). In general, P increased in the presence of acyclic derivatives, consistent with a decrease in membrane fluidity. This decrease was noticeably accompanied by an increase in k_2 and a decrease in R_0. The fact that k_1 was not affected by this change in a general property of the membrane suggested the proton leakage pathway, at least under energized conditions (*i.e.* active ATP hydrolysis occurring), may be localized in an area unaffected by FL treatment. Alternatively, the influence of the treatment may be removed by the energization process for example, by conformational changes in proteins.

DISCUSSION

The results described in this study provide further evidence to support our previous hypothesis (27, 29, 30) that the

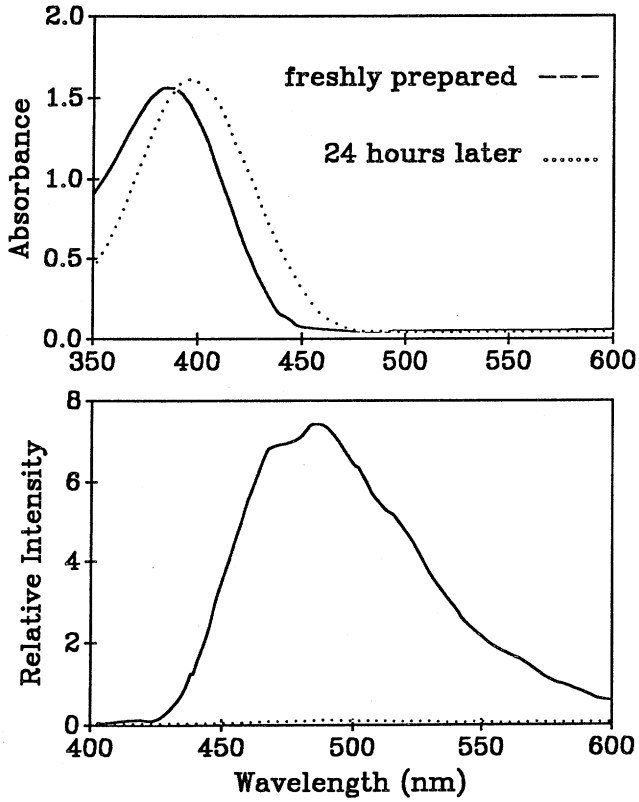

Figure 6. Comparison of spectroscopic properties between compounds II and III. The absorption (upper panel, solid line) and fluorescence (lower panel, solid line) spectra of fluorescamine derivative (II) with phenylalanine (Phe-FL) were recorded immediately after synthesis. The cyclization of II to form III was allowed to proceed in the aqueous acetone solution for 24 h at 22°C in the dark. The absorption (upper panel, dotted line) and fluorescence (lower panel, dotted line) spectra of cyclized derivative (III) were then recorded. For fluorescence measurements, the excitation wavelength was set at 390 nm. Similar spectroscopic changes were also observed with other fluorescamine-primary amine derivatives mentioned in text.

reorganization of energy transducing membrane such as that observed in mitochondrial inner membrane (24).

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